The Isolation of DNA from *Ehrlichia chaffeensis* Coming from Individual Ticks (*Amblyomma americanum*) Using Isocode Stix®

A THESIS SUBMITTED TO THE HONORS COLLEGE IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

For the degree

BACHELOR OF SCIENCE

By

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Advisor Robert R. Punga Date 4/22/02

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THESIS ABSTRACT

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This project was designed to determine the presence of *Ehrlichia chaffeensis* in the hemolymph individual *Amblyomma americanum* ticks using the Isocode Stix® method. The DNA was extracted from the Isocode Stix® and amplified using nested PCR. The PCR products were then visualized using a 1.5% ethridium bromide agarose-gel electrophoresis.

When the results from the Isocode Stix® method all came back negative, a CTAB-DNA extraction was performed on the carcasses of the same ticks used in the previous method. This was an attempt to check and see if the Isocode Stix® method was simply not sensitive enough. However, the results again came back all negative.

The results from this study showed that none of the 149 ticks collected were infected with *E. chaffeensis*. This is an unlikely result when compared to a similar study in which ticks were collected in the same county and approximately 6% of those ticks tested positive for *E. chaffeensis*. If, however, it is true that none of the ticks were actually infected, I would recommend trying this procedure again in the future on a larger sample of ticks.

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I would like to give a special thanks to Fresia Steiner, M.S. for all of her guidance and supervision throughout the entire project. I would also like to thank Dr. Robert Pinger for making this project possible.

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I. Introduction

Human monocytic ehrlichiosis is a tick-borne disease caused by *Ehrlichia chaffeensis*. *E. chaffeensis* is a gram-negative, obligate, intracellular bacteria belonging to the family Ricketsiaceae. The lone star tick, *Amblyomma americanum*, is the primary vector of *E. chaffeensis*. These ticks occur primarily in the southeastern and south central parts of the country. *Ehrlichia chaffeensis* was at first identified as *E. canis*, which causes disease in dogs. Further testing distinguished it from *E. canis* through the detection of minor differences in the 16S ribosomal subunit sequence. *E. chaffeensis* was first described independently of *E. canis* in 1987 (Maeda, Markowitz, Hawley, et al. 1987).

The symptoms of *E. chaffeensis* are quite similar to those of Rocky Mountain spotted fever (another rickettsial disease) which frequently leads to the initial misdiagnosis of *E. chaffeensis*. The incubation period for this illness is approximately nine days from the date of the tick bite. Initial symptoms include fever, chills, headache, muscle pain, and nausea. Although these symptoms are rather general in nature, infected individuals are often sick enough to seek medical attention. Infection with *E. chaffeensis* can be fatal, but the severity of the symptoms has proven to be dose and host dependent (Dumler and Bakken 1998). It is possible for people of all ages to become infected with *E. chaffeensis*; however, it is reported more frequently in the elderly due to an increasing decline in immunocompetence (Dumler and Bakken 1998).

The purpose of this project is to detect the presence of *E. chaffeensis* in samples of hemolymph (that was collected and stored on Isocode Stix®) of

individual *Amblyomma americanum* ticks. Negative test results could imply either the absence of *E. chaffeensis* or that the technique used for extracting the DNA was not sensitive enough to detect the bacteria. A positive test result indicates the presence of *E. chaffeensis* in an individual tick. If this method proves to be sensitive enough, it would mean that individual ticks could be tested for the presence of *E. chaffeensis* relatively quickly and easily. Previous techniques were only capable of detecting this bacterium in pools of ticks, and then only through a lengthy procedure of crushing, extraction, PCR, and electorphoresis. If the Isocode Stix® method can be perfected, the Public Health Entomology Laboratory could then offer this test to the citizens of Indiana.

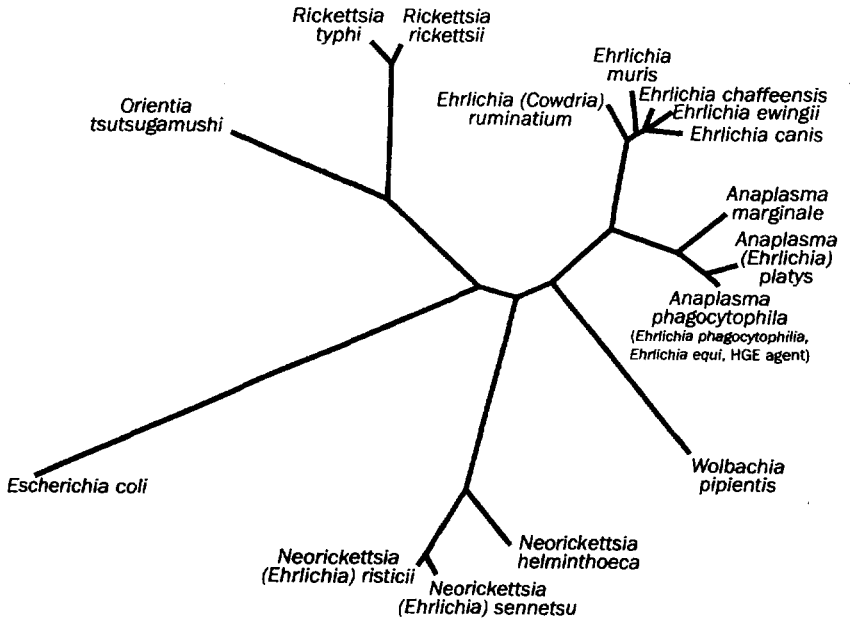
Literature Review

Ehrlichia chaffeensis

The genus *Ehrlichia* contains ten members, all of which are small (0.5-1.5 micrometers), non-motile, gram-negative, pleomorphic, obligate intracellular organisms. Deer are the established reservoir for *E. chaffeensis* (Lockhart et. al 1997). However, these organisms are transmitted through an arthropod bite into the skin. After having crossed the skin barrier, the organisms are phagocytized by circulating leukocytes. They then reproduce to form morulae which in turn rupture to release elementary bodies into circulation, where they infect other leukocytes. The type of leukocyte infected is one factor that differentiates the *Ehrlichia* species. *E. chaffeensis* primarily infects mononuclear cells (Fritz and Glaser 1998).

There are three distinct groups of *Ehrlichia* within the family Rickettsiaceae, and the relationship between these groups can be seen in the following cladogram (Dumler and Walker 2001).

Figure 1. Cladogram demonstrating the evolutionary relationships of Ehrlichia, Anaplasma, Wolbachia, Neorickettisa, Orientia, and Rickettsia species based on 16S rRNA gene sequences



The first record of disease caused by *Ehrlichia* occurred in 1935 among research dogs. Scientists then reproduced the disease in healthy dogs by inoculation with blood from the diseased dogs or homogenates of ticks (Donatien and Lestoquard 1937). The infectious agent was initially labeled *Rickettsia canis* but was later renamed *Ehrlichia canis*. *Ehrlichia* was then found to cause disease in many different veterinary species. *E. platys* and *E. ewingii* were also discovered to infect wild and domestic dogs. *E. equi* was found to infect horses and occasionally dogs, while *E. risticii* was discovered to be the agent causing Potomac Horse Fever (Holland et. al 1985). The following table represents the different groups within the family Richettsiaceae (Fritz and Glaser 1998).

Table 1. THREE GENOGROUPS IN THE FAMILY RICKETTSIACEAE CONTAINING MEMBERS OF EHRLICHIEAE

	Species	Vertebrate Hosts	Leukocytotropism	Vector	Distribution
I.	*Ehrlichia canis*	Dogs	Mononuclear cells	*Rhipicephalus sanguineus*	Worldwide
	E. chaffeensis	Humans	Mononuclear cells	*Amblyomma americanum, Dermacentor variabilis*	United States
	E. ewingii	Dogs	Granulocytes	*A. americanum*	United States
	E. muris	Vole	Mononuclear cells	Unknown	Japan
	Cowdria ruminatium	Cattle, sheep, goats, antelope	Vascular endothelium	*Amblyomma* spp.	Sub-Saharan Africa, Caribbean
II.	*E. phagocytophila*	Sheep, cattle, bison, deer	Granulocytes	*Ixodes ricinus*	Northern Europe
	E. equi	Horses, dogs	Granulocytes	*I. pacificus*	United States
	HGE agent	Humans	Granulocytes	*I. scapularis, I. pacificus*	United States
	E. platys	Dogs	Thrombocytes	Ticks *(Rhipicephalus?)*	Southern United States, Southern Europe
	Anaplasma marginale	Cattle	Erythrocytes	*Dermacentor*	Worldwide
III.	*E. sennetsu*	Humans	Mononuclear cells	Unknown	Japan, Malaysia
	E. risticci	Horses	Mononuclear cells	Unknown	North America
	Neorickettsia helminthoeca	Dogs	Mononuclear cells	*Nanophyetes salmincola*	North American Pacific Coast
	N. elokominica	Dogs	Mononuclear cells	*N. salmincola*	North American Pacific Coast

The first recorded case of human ehrlichiosis in the Western Hemisphere occurred in 1986. The patient presented with flu-like symptoms ten days after obtaining a tick bite. The laboratory results from this patient included leucopenia, thrombocytopenia, elevated hepatic enzymes and creatinine, and evidence of disseminated intravascular coagulation (Maeda et. al 1987). The patient also demonstrated an acute titer of *E. canis* antigen. In the years following the first reported case, many more cases of the human monocytic ehrlichiosis (HME) have been reported. Further testing using a method based on amplification and sequencing of the 16S rRNA gene, determined that the agent causing human ehrlichiosis was significantly different enough from *E. canis* that it warranted its own classification. This new rickettsial organism was named *E. chaffeensis* in 1987 (Anderson et al. 1991).

Amblyomma americanum

The genus *Amblyomma* comprises approximately 100 species of ticks. These ticks are generally characterized by a large body size and by being highly ornamented with long mouth parts, eyes, and festoons (Sonenshine 1991). Most species of *Amblyomma* are distributed in tropical climates, but two species occur in significant numbers in the southern United States, *Amblyomma americanum* and *Amblyomma maculatum.*

Figure 2. The female can be seen on the left and the male is displayed on the right.

Ticks in the genus *Amblyomma* have a three-host life cycle that involves parasitizing a wide range of different hosts (Sonenshine 1991). This exposure to a wide range of hosts increases the risk of the tick becoming infected and transmitting disease. The larvae and nymphs of *A. americanum* feed on small and medium-sized mammals,

while the adults prefer larger mammals such as deer, dogs, and coyotes (Means and White 1997). However, white-tailed deer can serve as an important host for all three life stages and are suspected to be the major reservoir. In addition, all stages also readily feed on humans (Means and White 1997). After infection, ticks generally transmit the disease to a new population, but humans usually serve as dead end hosts for pathogens (Sonenshine 1991).

Diagnosis

The severity of infection with *E. chaffeensis* ranges from sub-clinical to fatal, fatalities are invariably limited to immunocompromised people. Symptoms generally occur approximately nine days after tick exposure. This infection generally presents with the abrupt onset of fever, headache, myalgia, and chills. Less common symptoms also include nausea, vomiting, diarrhea, abdominal pain, coughing, and confusion (Fishbein, Dawson, and Robinson 1994)). A rash also occurs in about one-third of the patients and is more common in children. The rash may not occur until several days into the illness, is short-lived, and is not associated with the site of tick attachment (Walker and Dumler 1996). Some other more severe complications of ehrlichiosis include respiratory and renal failure, opportunistic infections, and hemorrhage (Fishbein, Dawson, and Robinson 1994). Table 2 summarizes the clinical symptoms of patients with ehrlichiosis (Fritz and Glaser 1998).

Table 2. CLINICAL FEATURES OF PATIENTS WITH EHRLICHIOSIS

Most Frequent	Frequent	Occasional
Fever	Nausea	Toxic shock syndrome
Headache	Cough/dyspnea	Myocardial complications/involvement (e.g., left ventricular dilatation)
Myalgia	Rash	
Shaking chills	Vomiting	Brachial plexopathy
Malaise	Diarrhea	Prolonged fever
History of tick bite	Abdominal pain	Hypotension
	Confusion	Lymphadenopathy
	Arthralgias	Pharyngitis
	Anorexia	Conjunctivitis
		Acute respiratory distress syndrome

The primary laboratory characteristics of ehrlichiosis include leucopenia, thrombocytopenia, and elevated hepatic transaminases. "One-third to one-half of patients have an elevated blood urea nitrogen and creatinine, often two to three times normal. The hepatic enzymes frequently normalize after several days of therapy" (Ratnasamy, et. al 1996). Table 3 depicts some of the laboratory features of patients with ehrlichiosis (Fritz and Glaser 1998).

Table 3. LABORATORY FEATURES OF PATIENTS WITH EHRLICHIOSIS

Most Frequent	Frequent	Occasional
Leukopenia	Anemia	Elevated CPK
Lymphocytopenia	Morulae	Elevated bilirubin
Elevated aspartate aminotransferase	CSF pleocytosis	
Elevated alanine aminotransferase	CSF elevated protein	
Thrombocytopenia	Fibrin split products	
	Hyponatremia	
	Elevated ESR	
	Elevated BUN and creatinine	

Diagnostic tests are not widely available, but can be requested through health departments, the Centers for Disease Control and Prevention (CDC), and a few private research laboratories (Dumler and Walker 2001). Serology has proven to be the most sensitive confirmatory test. The titers are absent early in the infection, but become detectable by the third week of illness. "Diagnosis of HME and HGE is made by indirect immunoflourescent antibody (IFA) detection using *E. chaffeensis* and *E. equi* antigens, respectively, and is based on a four-fold rise or fall in titer. Because these organisms differ antigenically, it is necessary to test for both HME and HGE" (Fritz and Glaser 1998). Blood smears or buffy coat preparations would demonstrate the presence or absence of morulae which are characteristic of ehrlichiosis. Although the smears are not sensitive enough, they help establish the diagnosis and are available immediately. Figure 3 shows a picture of typical ehrlichial morulae within the cytoplasm of the infected cells (Houpikian and Raoult 2002).

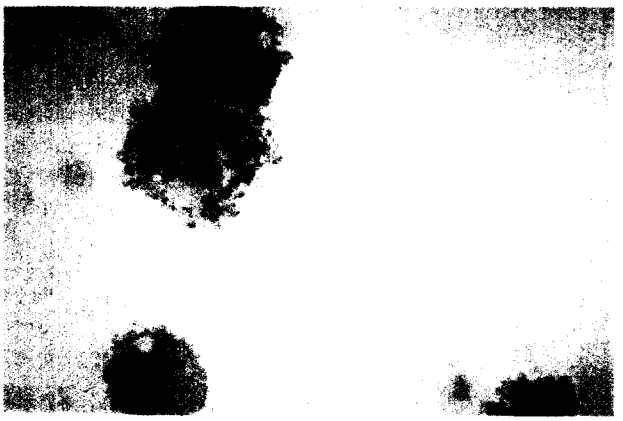


Figure 3. Canine monocytes (DH82) heavily infected with E. chaffeensis

Other frequently used diagnostic techniques include immunohistochemistry, culture, and polymerase chain reactions. Immunocytology and immunihistology are also capable of

confirming that the "intraleukocytic inclusions" seen on the blood smears are *Ehrlichia* morulae.

Polymerase Chain Reaction

Polymerase chain reaction, commonly known as PCR, is a method of detecting the presence of foreign DNA incorporated into the tick DNA. This is done using specific primers that target the 16S rRNA gene and then amplifying it. The 16S rRNA gene of *E. chaffeensis* exists in a few copies within the genome, occurring in tandem repeats. Each copy of this region contains both conserved and non-conserved regions (Burket et al., 1998). Non-conserved regions display more frequent sequence variations between species, and thus, these are the regions targeted using species-specific primers when distinguishing between species. The targeted regions are amplified during the PCR process and can be used in further testing.

Electorphoresis

An ethidium bromide agarose gel electrophoresis can provide a visual image of the DNA amplified during the polymerase chain reaction. Gel-loading buffer is added to the PCR products and allowed to run in the electrophoresis. The gel is then examined under ultraviolet lighting to observe the DNA bands. The presence of *E. chaffeensis* can be determined by the locations of specific bands.

Materials and Methods

DNA extraction using Isocode Stix ®

DNA was extracted from individual ticks using the Isocode Stix® method. The foreleg of the tick was cut and a drop of hemolymph was placed on the tip of the Isocode Stix® and then stored. The tip of the stick was placed in a tube and mixed with 100µl of deionized water. The tube was briefly centrifuged and then placed in a heating block at 100° Celsius for 15 minutes. Then pulse vortex 60 times, and centrifuge again briefly. After the last centrifugation, remove the tip of the stick using forceps. Squeeze the tip very well on the side of the tube to remove all the excess water. The paper can then be discarded and the solution is used for PCR.

Approximately 70 samples into the experiment, the extraction was changed slightly. All steps of the extraction remained the same except the removal of the tip of the Isocode Stix®. In this case, the PCR was run with the tip of the stick in the solution in an attempt to maximize the amount of DNA present.

DNA extraction on pools of ticks

DNA was extracted from pools of ticks using the cetlytrimethylammonium-bromide (CTAB) method. Tick samples, each containing four ticks, were homogenized in the corner of a plastic bag by crushing them with a hammer several times. The bag was then washed out using 400µl of CTAB isolation buffer. The homogenate along with the tick skeleton were placed in a microcentrifuge tube and placed in a 65° Celsius water bath for 30 minutes. After incubation was completed, 200µl of phenol and 200µl of chloroform: isoamyl alcohol were added to each tube. After which each tube was briefly vortexed and then centrifuged for 10 minutes at 10,000 rpm. Then the aqueous solution from each tube was then placed in a new microcentrifuge tube, while 400µl of non-CTAB

extraction buffer was added to the organic matter in tube. The tubes were again vortexed followed by centrifugation for 10 minutes at 10,000 rpm. The organic solution was discarded. The aqueous solution from the tubes was then combined with the first aqueous solution and 800µl of chloroform: isoamyl alcohol was added to each tube. Next the solutions were mixed by shaking and then again centrifuged for 10 minutes 10,000 rpm. Again, following centrifugation, the organic solution is discarded. The aqueous phase was placed in clean microcentrifuge tubes. The DNA was then precipitated using 534µl of isopropanol. The solution was then incubated at -20° C overnight. The supernatant was then decanted and the pellet was washed with 80% ethanol to remove the remaining CTAB and isopropanol, and finally, the pellet was resuspended in 50ul of TE.

Template preparation and Nested PCR

Two rounds of PCR were performed on a Perkin Elmer 2400 thermal cycler. The extracted DNA was used as a template for nested PCR amplification of the 16S rRNA gene of *E. chaffeensis*. 15µl of the extracted DNA was combined with 35µl of a master mix containing deionized water, 10 x buffer, 25mM $MgCl_2$, 10 mM of each of the dNTPs, and Taq Polymerase. The primers used in the first round were ECB and ECC. These primers amplify a DNA fragment common in all *Ehrlichia* species as well as a few other bacterial species (Dawson et al., 1994). The overall reaction mixture was 50µl. The reaction was run for 30 cycles using the following temperature profile: 15s at 94°C, 30s at 48°C, and 30s at 72°C, with a final extension of 5 minutes at 72°C.

For the second round of PCR, 2µl of the product from the first round was combined with the same reaction mixture as above with the addition of 6% DMSO

(dimethlysulfoxide) and the use of HE1 and HE3 primers instead of ECB and ECC. These primers amplify a 389 base pair fragment specific to *E. chaffeensis*, which is contained within the larger PCR fragment obtained from the first amplification (Steiner, et. al 1999).

Gel electorphoresis of PCR products

The final products of the nested PCR reactions were separated through a 1.5% agarose gel. This gel was dyed with ethidium bromide (EtBr). This dye intercalates between the DNA bases and allows for visualization on an UV transilluminator. 10 µl of loading dye (gel buffer) was added to each of the PCR products, and then 14µl of this mixture was loaded into the wells of the agarose gel. The gel was run in a 1 x TBE buffer at 60 volts for 1.5 hours.

Results

In order to verify that the Isocode Stix® method would work, a sample model was run using DH82 – *E. chaffeensis* infected cells. Varying concentrations were used, providing a sort of calibration curve. The first well (the one to the far left) contained a negative control. The second well contained a 1:100 dilution. The third well was a 1:10 dilution and the fourth well was not diluted and contained 2µl of the sample. The fifth well was left empty, and a marker (phiX174) was run in the sixth well. A photograph of the electrophoresis shows a continually stronger positive result as the samples become less dilute.

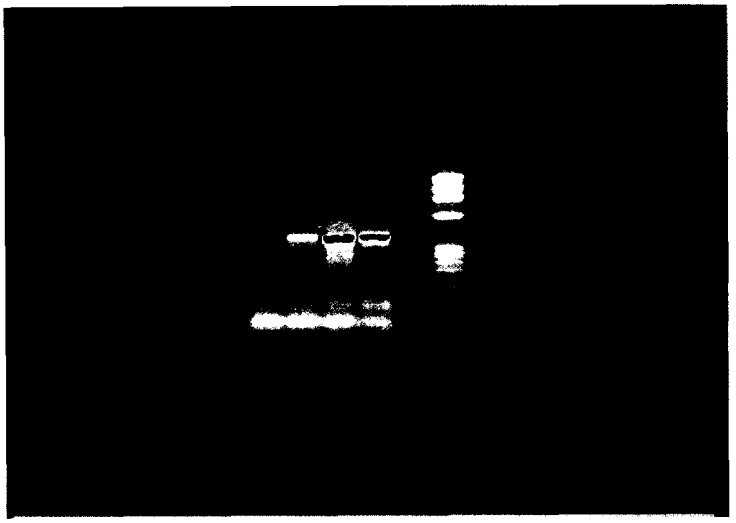


Figure 4. Electrophoresis using varying dilutions of DH82

After concluding that the Isocode Stix® method would work, it was performed on 77 samples from *Amblyomma americanum* with zero positive results. Taking into consideration that the samples may have been too dilute, the method was slightly modified. Instead of squeezing and removing the tip of the Isocode Stix®, it was left in the microcentrifuge tube and run through the first round of nested PCR, including a "hotstart" step of 5 minutes at 95°C. This modification was performed on 72 samples. In this round of testing, there were zero positive results.

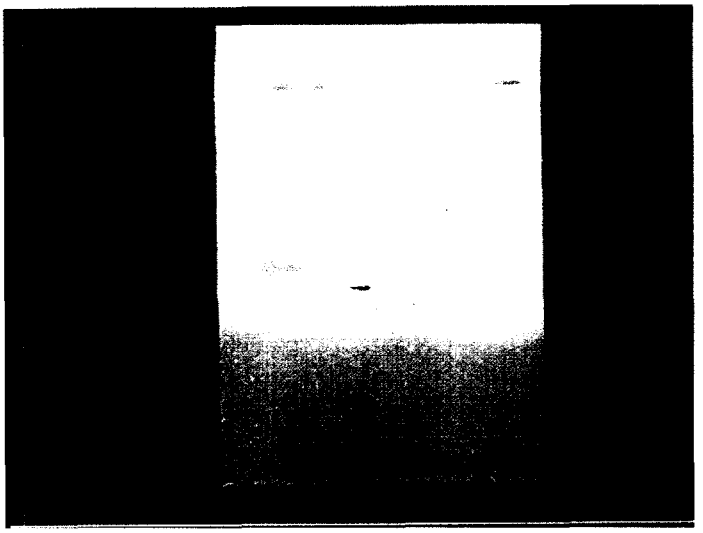


Figure 5. Electrophoresis ,showing all negative results, using the Isocode Stix(R) method.

CTAB extractions were then performed on the carcasses of pools of ticks used in the Isocode Stix® method. This method was performed on all 149 ticks previously used. Again, there were zero positive results. This electrophoresis was run with a marker in the first well and a positive control in the sixth well.

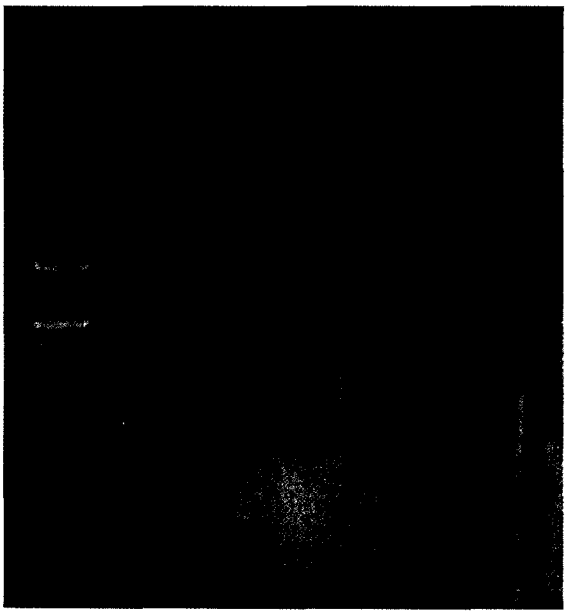


Figure 6. Electrophoresis using the CTAB extraction method.

Discussion

Isocode Stix® are currently being used in labs for many purposes including: HLA typing, forensic studies, paternity testing, epidemiological studies, and many molecular-based research projects. In this case, it would have provided the Public Health Entomology Laboratory an efficient and inexpensive method of testing individual ticks for *E. chaffeensis.*

The project did not work as anticipated for two possible reasons. The first and probably most likely, is that the samples were too dilute. There was very little hemolymph on the Isocode Stix® to begin with, and so mixing it with as much water as was required really diluted the sample that was there. The CTAB extractions, done on pools of the same ticks used in the Isocode extractions, were an attempt to determine if *E. chaffeensis* was in fact present. If the CTAB results had come back positive, it would have proven that the Isocode Stix® method was simply not sensitive enough to detect the presence of *E. chaffeensis.*

The CTAB extractions, however, did not come back positive. One reason for this is that *E. chaffeensis* is present in the hemolymph of ticks and the hemolymph was all removed and placed onto the Isocode Stix®. This would mean that even if the ticks were infected with *E. chaffeensis,* they would not test positive through either method of extraction because the hemolymph was removed from the carcass and the Isocode Stix® method was not sensitive enough.

There is also the very remote possibility that none of the ticks were actually infected with *E. chaffeensis.* This is not very likely when comparing this data to that of

others who collected ticks in the same county as the ticks used in this experiment. These ticks were collected in Warrick County as were those in the previously mentioned project. In that project, 227 ticks were collected and tested for infection with *E. chaffeensis*. Of these 227 ticks, thirteen of them tested positive. This is approximately 6% of the ticks collected (Burket, Vann, Pinger, Chatot, and Steiner 1998). In comparing that data with that data from this project, in which 149 ticks were collected from the same county and all came back negative when tested for *E. chaffeensis*, it does not seem likely that the ticks in this project were just not infected with *E. chaffeensis*.

Although this project did not prove to be the efficient and inexpensive tool it was hoped to be, I do not feel that it was time wasted. There is much to be learned from this experiment. The Isocode Stix may yet prove to be an effective tool if a method of concentrating the DNA in the hemolymph is developed or finding a way of extracting more of the hemolymph from the tick is found. However, if it is true that the ticks were not infected, then I would recommend trying this procedure in the future on a larger sample of ticks.

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